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ORIGINAL ARTICLE

The Effect of the *Cyp19a1* Gene Methylation Modification on Temperature-dependent Sex Determination of Reeves' Turtle (*Mauremys reevesii*)

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Abstract Temperature-dependent sex determination (TSD) is a type of environmental sex determination in which the sex of the embryos depends on the ambient temperature; however, the molecular mechanisms governing this process remain unknown. Aromatase, encoded by the *cyp19a1* gene, which converts androgens into estrogens in animals, was considered to be the key gene for TSD. In this study, the 5'-flanking region of the *cyp19a1* gene in Reeves' turtle (*Mauremys reevesii*) was cloned, and the promoter region was identified using the luciferase reporter assay. Then the eggs of Reeves' turtle were incubated at different temperatures (26°C: male-biased temperature; 29°C: non-sex-biased temperature and 32°C: female-biased temperature). During the thermosensitive period, the adrenal kidney gonad complexes (AKG) were sampled. DNA methylation analysis of the AKG samples showed that the promoter region of the *cyp19a1* gene was significantly de-methylated in the female-biased temperature regime ($P<0.01$). Quantitative analysis of the *cyp19a1* gene and estrogen by qPCR and Elisa assay showed that the expression level of the *cyp19a1* gene and estrogen content were both upregulated significantly at the female-biased temperature ($P<0.01$). These results indicated that the de-methylation response of the *cyp19a1* gene to incubation temperature, especially at the female-biased temperature, could lead to temperature-specific expression of aromatase and increased estrogen levels, which may further determine gonadal development in Reeves' turtle. These findings provide insights into the genetic mechanisms underlying TSD.

Keywords Reeves' turtle, *Mauremys reevesii*, temperature-dependent sex determination (TSD), *cyp19a1* gene, methylation

1. Introduction

Sex determination mechanisms in vertebrates are typically divided into genotypic sex determination (GSD) and environmental sex determination (ESD). In GSD, gonadal sex is determined by heritable genetic elements at the moment of fertilization, whereas in ESD, gonadal sex is determined by environmental factors after fertilization. Temperature-dependent sex determination (TSD) is the main form of ESD (He *et al.*, 2009; Du and Sun, 2012). The gender of individuals in species exhibiting TSD depends on the ambient temperature during embryonic incubation. In the middle one-third of embryonic development, environmental temperature irreversibly determines gonadal sex; this critical period of incubation

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is known as the thermosensitive period (TSP) (Bull, 1980). However, the molecular mechanisms governing this process remain unknown.

TSD is most common in fish and reptiles; currently, a number of relevant genes regulating sex determination in TSD have been found. In general, these genes can be divided into testicular-determining genes, including *DMRT1*, *Sox9* and *SF-1*, and ovarian-determining genes, including *FOXL2* and *cyp19a1* (Shoemaker *et al.*, 2007). Aromatase, the product of the *cyp19a1* gene, is a steroidogenic enzyme that irreversibly converts androgens into estrogens, and therefore plays a central role in ovarian development (Simpson *et al.*, 1994). In fish and reptiles, masculinization induced by temperature is related to the inhibition of expression of *cyp19a1*. Exposure to female-biased temperatures is accompanied by gonadal *cyp19a1* upregulation, whereas exposure to male-producing temperatures is accompanied by *cyp19a1* suppression (Ospina-Álvarez and Piferrer, 2008; Caruso *et al.*, 2015). These findings indicate that temperature can regulate transcription of the *cyp19a1* gene, causing the previously undifferentiated gonads to develop into ovaries or testes.

It is well established that epigenetic mechanisms can allow an organism to respond to environmental changes, and integrate environmental and genetic information to produce a particular phenotype (Jaenisch and Bird, 2003; Piferrer, 2013). In particular, DNA methylation, which often occurs in promoter or regulatory regions, is capable of inhibiting gene transcription to adjust the function of genes (Razin and Riggs, 1980). For example, a female-biased temperature during the TSP has been found to cause demethylation at the promoter region of *cyp19a1*, and result in the temperature-specific expression of aromatase in red-eared slider turtles (*Trachemys scripta elegans*) and alligators (*Alligator mississippiensis*) (Matsumoto *et al.*, 2013; Parrott *et al.*, 2013). It is speculated that methylation may be one of the important regulatory mechanisms in TSD. Therefore, further investigation of the response of the *cyp19a1* gene to ambient temperatures in various organisms is important in understanding the regulation of TSD.

In China, Reeves' Turtle (*Mauremys reevesii*) is one of the common Chelonia species (Hou, 1985), and belongs to TSD Ia, in which there is a male bias at low temperatures and a female bias at high temperatures (Li, 2002). In this study, the 5'-flanking region of the *cyp19a1* gene in Reeves' Turtle was cloned, and the promoter region was identified using the luciferase reporter assay. DNA methylation variation in the *cyp19a1* gene promoter region, the expression level of the *cyp19a1* gene, and the estrogen content in gonadal samples during the TSP were investigated. These data provide insights into the genetic mechanisms underlying TSD, and may expand our current understanding of the gene regulatory pathways operating during vertebrate sex determination.

2. Materials and Methods

Ethics statement All research involving animals was performed under approval from the Animal Ethics Committee at the Guangdong Institute of Applied Biological Resources.

Sample collection Reeves' turtle eggs, freshly laid and with fertilization spots, were collected from Guangdong LVCA Industry Co., Ltd. (Guangzhou Guangdong, China). The eggs were randomly allocated into plastic containers filled with moist vermiculite (Du *et al.*, 2007). Containers were placed into 3 homothermal incubators (Shanghai JingHong Laboratory Instrument Co. Ltd., China) set at 32°C (female-biased temperature), 29°C (non-sex-biased temperature) or 26°C (male-biased temperature) respectively (Li, 2002). The temperature inside the incubators was monitored daily using thermistor probes (Deli Group Co. Ltd., Ningbo City, China).

According to the standardized staging criteria, based on morphological features of the turtle embryo (Tan *et al.*, 2001; Greenbaum, 2002; Yang *et al.*, 2011), Reeves' turtle embryos were monitored for developmental stage by dissection of 2–3 eggs each day. Because gonadal tissues had not clearly differentiated, the adrenal kidney gonad (AKG) complex was sampled until embryos developed to stage 16, at which point the embryonic gonads were responsive to ambient temperature (Matsumoto *et al.*, 2014).

Thirty AKG complexes from each temperature regime were sampled, and were snap-frozen in liquid nitrogen, then stored at –80°C prior to further analysis. The rest of the eggs were allowed to hatch. The gender of hatchlings was established based on the sexual dimorphism of the gonads. All efforts were made to minimize suffering of the animals during the procedures.

Cloning and sequence analysis of the 5'-flanking region of the *cyp19a1* gene Genomic DNA, isolated from the tail of a Reeves' turtle, was used to clone the 5'-flanking region of the *cyp19a1* gene. By homology analysis of species related to the Reeves' turtle, including *Chelonia mydas, Trachemys scripta elegans* and *Chrysemys picta*, a set of homologous primers were

used to clone the 5'-flanking region of the *cyp19a1* gene of the Reeves' turtle. The primer sequences were: 5FR-F: 5'-AGATAACAGTATCTGCCTCC-3', 5FR-R: 5'-AAGATAGTTTGCCTGGGTC-3'. The PCR conditions were: 5 min at 95°C, followed by 34 cycles of 30 s at 95°C, 30 s at 59°C, 105 s at 72°C and a final extension of 10 min at 72°C.The amplification products were gel purified and cloned into pGEM-T (Takara, Dalian, China) for sequencing.

Promoter activity analysis by luciferase reporter assay The transcriptional start site and transcription factor-binding sites of the 5'-flanking region of the *cyp19a1* gene were predicted using Promoter 2.0 and the TRANSFAC 6.0 database (Si *et al.*, 2016). Based on the predictions, the putative promoter region (PPR) of the *cyp19a1* gene was PCR amplified. The primer sequences were: PPR-F:5'-gcgtgctagcccgggctcgagCAGCAAGGAACCCTTCTC-3', PPR-R:5'-cagtaccggaatgccaagcttCATCACTTCTGGCACCAC-3'. The PCR conditions were: 5 min at 95°C, followed by 34 cycles of 30 s at 95°C, 30 s at 55°C, 75 s at 72°C and a final extension of 10 min at 72°C.

After gel purification, the plasmid of pGL3-PPR was constructed using a ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China) by recombining the PPR DNA fragment into the pGL3-basic vector. Then the plasmid of pGL3-PPR was used to analyse promoter activity using the luciferase reporter assay.

The pGL3-PPR and PRL-TK plasmids were collectively transfected into HEK 293T cells, in a ratio of 25:1. The pGL3-basic empty plasmid was used as a negative control and cells with no plasmids were used as a blank control. All transfections were carried out in triplicate. After transfection, the cells were harvested at a 90% degree of convergence, and a microporous luminescence analyzer was used to detect promoter activity (Oshima *et al.*, 2006).

DNA methylation measurement of the *cyp19a1* promoter by bisulfite sequencing The genomic DNA of 10 AKG complexes sampled from each incubation temperature was isolated using a Rapid Animal Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China), and the DNA from each incubation temperature was pooled together to construct a sequencing library. DNA samples were sodium bisulfite-modified using the Mag-DNA Modification Kit (Sangon Biotech, Shanghai, China) following the manufacturer's instructions.

Using the online software Methprimer, we predicted CpG sites in the region of the promoter between –352 and –3 bp [relative to the position of putative transcription start site (TSS) counted as +1].

The sequencing primers were F: 5'-TAGAAATTTGGTATTTTGTTTGGAG-3', R: 5'-TTTCAAAATACTAAAACACCTTTAATC-3'. The PCR conditions were: 4 min at 98°C, followed by 20 cycles of 45 sec at 94°C, then 45 sec from 66°C, taking readings at 0.5°C decrements to 56°C, 1 min at 72°C, and a final extension of 10 min at 72°C. The PCR products were gel purified, and cloned into the pGEM-T Vector. A total of 30 clones per library were sequenced (Si *et al.*, 2016).

Gene expression analyses of the *cyp19a1* gene by quantitative RT-PCR Three AKG complexes from each incubation temperature were pooled together as a biological repetition, and three biological repetitions from each incubation temperature were used to test the gene expression of the *cyp19a1* gene.

Total RNA was isolated from the samples using RNAiso reagent (TaKaRa, Dalian, China). The purity and concentration of the RNAs were checked by UV spectrophotometry and the RNAs' integrity was checked by electrophoresis in a 1% agarose gel. Reverse transcription was performed with a PrimeScript™RT reagent Kit (TaKaRa, Dalian, China), following the manufacturer's instructions.

Quantitative RT-PCR It was performed with an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clare, CA) using an SYBR Premix Ex Taq™II Kit (Takara, Dalian, China). The protein phosphatase 1γ (PP1) was used as the endogenous reference (Ramsey *et al.*, 2007). The primer sequences were: *cyp19a1*-F: 5'-ATCATTCTGAACATTGGACG-3', *cyp19a1*-R:5'-TGGCTGAAAGTAGCGAGA-3'; PP1-F:5'-CAGCAGACCCTGAGAACTTCTTCCTGCTG-3', PP1-R: 5'-GCGCCTCTTGCACTCATCAT-3'.

A total volume of 12.5 μl contained 6.25 μl of 2× SYBR Premix Ex Taq, 1 μl of diluted cDNA and 0.5 μl of each primer, with RNase-free water to 12.5 μl, and the qPCR conditions were as follows: 95°C for 30s, followed by 40 cycles at 95°C for 5 s and 60°C for 1 min. A dissociation curve was performed at 95°C for 1 min, 55°C for 30 s, taking readings at 0.5°C increments until reaching 95°C for 1 min, to test for nonspecific amplification. Each sample was run in triplicate. Dates for each qPCR plate were automatically assigned by the MXPRO software (Agilent), and the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Bieser and Wibbels, 2014).

Determination of estrogen levels by ELISA assay Three AKG complexes from each incubation temperature were pooled together as a biological repetition, and three biological repetitions from each incubation temperature

were used to test the estrogen content. The samples were homogenized with 0.01 mol/L PBS (Pieau *et al.*, 1982). Then the sample homogenates were centrifuged to obtain the supernatant. Estrogen (E2) levels were measured using an enzyme-linked immunosorbent assay (Fish-ELISA KIT, Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China), according to the manufacturer's protocol. The optical density (OD) was determined using a PerkinElmer VICTOR3 (PerkinElmer, Waltham, MA) at a wavelength of 450 nm.

Statistical analysis Chi-Square tests were used to determine the influence of the incubation temperature on the sex ratios and methylation status modification of the *cyp19a1* promoter. One-way ANOVA tests were used to analyze significant differences in the *cyp19a1* promoter activity, gene expression, and estrogen content, using SPSS 13.0 (SPSS, Chicago, IL). To identify correlations between the DNA methylation treatment, gene expression, and estrogen content, Spearman tests were performed using SPSS 13.0. A *P*-value of less than 0.05 was considered to be statistically significant.

3. Results

Promoter activity found in –777 to +77 bp of the *cyp19a1* gene A total 1624 bp of the 5'-flanking region of the *cyp19a1* gene was successfully PCR amplified. The putative transcription start site and TATA box was predicted at 14 bp and 155 bp upstream of the translation start codon (ATG). Several sex determination-related transcription factor binding sites were also predicted, including the steroidogenic factor 1 (Sf1), fork head domain factors (Fox), SRY-like HMG box (Sox) and Wilm's tumor-associated gene (WT1), and they were found to be intensively distributed in the –777 to +77 bp of the 5'-flanking region (putative promoter region, PPR) (Figure 1A). The luciferase reporter assay showed that the PPR displayed high promoter activity (P<0.01, one-way ANOVA) (Figure 1B), confirming that this region contained a promoter.

Hatchling sex was significantly affected by incubation temperatures in Reeves' turtle The hatching results showed that a total of 78, 51 and 59 eggs were successfully hatched at 26°C, 29°C, and 32°C, respectively. Thirty hatchlings from each incubation temperature were randomly selected for sex identification. The female ratio was 3.3%, 36.6% and 100% for the 26°C, 29°C and 32°C groups, respectively (Figure 2), suggesting that the incubation temperature significantly affects hatchling sex in Reeves' turtle (P<0.01).

DNA methylation levels of the *cyp19a1* promoter are influenced by incubation temperature There are three CpG sites in the *cyp19a1* promoter region, respectively located at –73 bp, –193 bp and –326 bp relative to the TSS (Figure 1A). The methylation patterns of the three CpG sites were evaluated by bisulfite sequencing. The results showed that the overall percentages of DNA methylation were 87.8%, 85.6% and 71.1% at 26°C, 29°C and 32°C, respectively. The Chi square test showed that the DNA methylation at 32°C was significantly lower compared to the 26°C (P<0.01) and 29°C (P<0.05) groups (Figure 3A). The DNA methylation in each CpG

Figure 1 The sequence analysis and promoter activity validation of the putative promoter region (–777 to +77) in the *cyp19a1* gene. (A) CpG dinucleotides are shown in red font. Putative binding sites for relative transcription factors (TFBs) are indicated in colored boxes. The translation initiation codon "ATG" is highlighted in bold and the putative transcription start site (TSS, +1) is indicated with an arrow. The primer pairs used for DNA methylation analysis are underlined. (B) HEK293T cells were transfected with pGL3-PPR promoter and pGL3-basic control vectors. Values are indicated as mean + SEM. Double asterisk denotes a highly significant difference (P<0.01, one-way ANOVA).

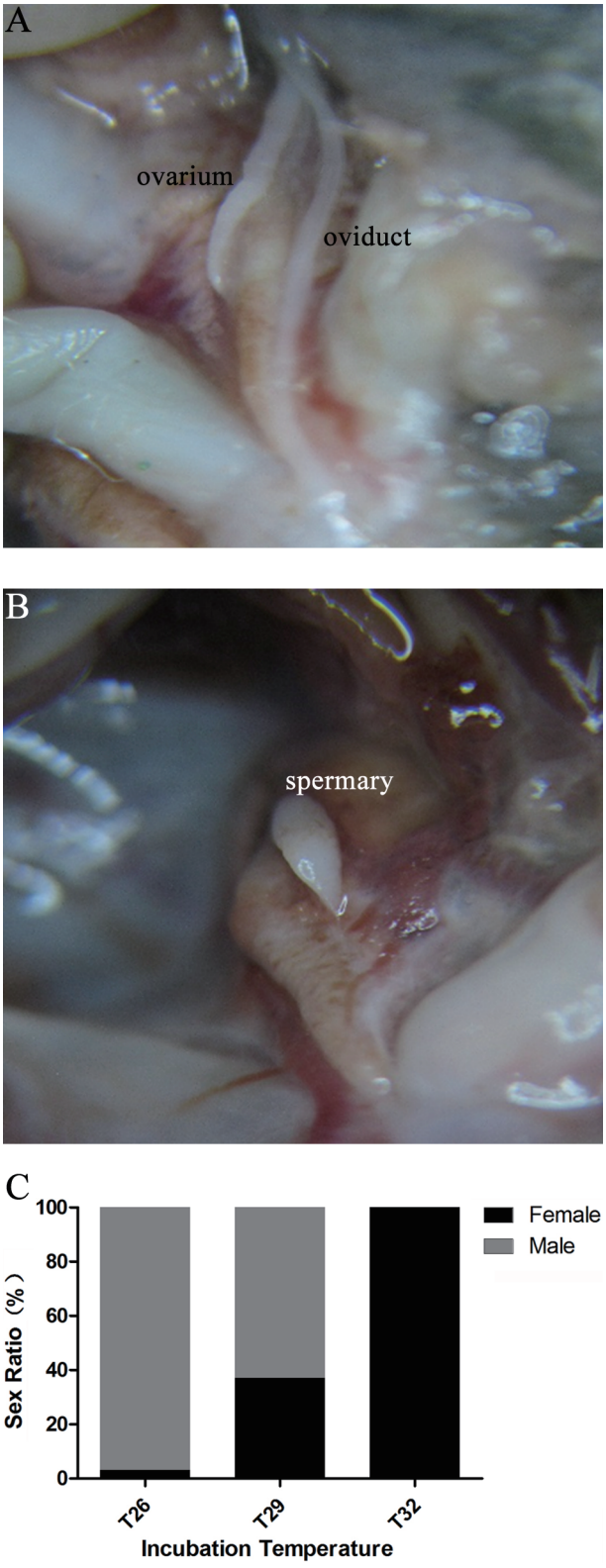

Figure 2 The effects of different incubation temperatures on sexual development in Reeves' turtles (A) The ovarium and oviduct of female hatchlings. (B) The spermary of male hatchlings. (C) The effects of different incubation temperatures on sex ratio in Reeves' turtles. Double asterisk indicates a highly significant difference (P<0.01).

site also experienced a downward trend with increasing incubation temperature, in which the –193 site was most changed (Figure 3B).

Expression of *cyp19a1* and estrogen content depend on the incubation temperature Quantitative RT-PCR showed that expression of the *cyp19a1* gene was upregulated as the incubation temperature increased, and the expression level at 32°C was significantly higher than at 26°C or 29°C (P<0.01) (Figure 4A).

Estrogen levels were 63.39 ng/L, 74.56 ng/L and 89.02 ng/L in the AKG complexes at 26°C, 29°C and 32°C, respectively, and statistical analysis showed that the estrogen content was significantly different between incubation temperatures (Figure 4B).

DNA methylation level of the *cyp19a1* promoter, expression of *cyp19a1* and the estrogen content were correlated with the sex ratio changes caused by incubation temperatures The CpG methylation level of the *cyp19a1* promoter showed a significant negative relationship with gene expression (P<0.01) (Figure 5A). There was a significant positive relationship between *cyp19a1* expression and estrogen content at different incubation temperatures (P<0.01) (Figure 5B), and a significant positive relationship between estrogen content and sex ratios (P<0.01) (Figure 5C).

4. Discussion

Accumulating evidence indicates that the sexual differentiation of gonads is controlled by epigenetic regulation of the sex-related genes (Piferrer, 2013). For example, the sex of European sea bass is controlled by both environmental and genetic factors (Vandeputte *et al.*, 2007). Males of the European sea bass have double the DNA methylation levels of females in the *cyp19a1* promoter, while temperature-induced masculinization is also associated with the methylation level of the *cyp19a1* gene promoter region, with an observed inverse relationship between methylation levels and gene expression (Navarro-Martín *et al.*, 2011). In the red-eared slider turtle, a species with TSD, exposure of embryos to female-producing temperatures leads to a decrease in DNA methylation at the aromatase promoter region, which corresponds to the expression of the aromatase gene increasing during gonad development. However, whether the epigenetic regulation of the sex-related genes serves as a conservative regulation pattern in sex determination, especially in TSD animals, needs more experimental verification.

In this study, the promoter region of the *cyp19a1* gene

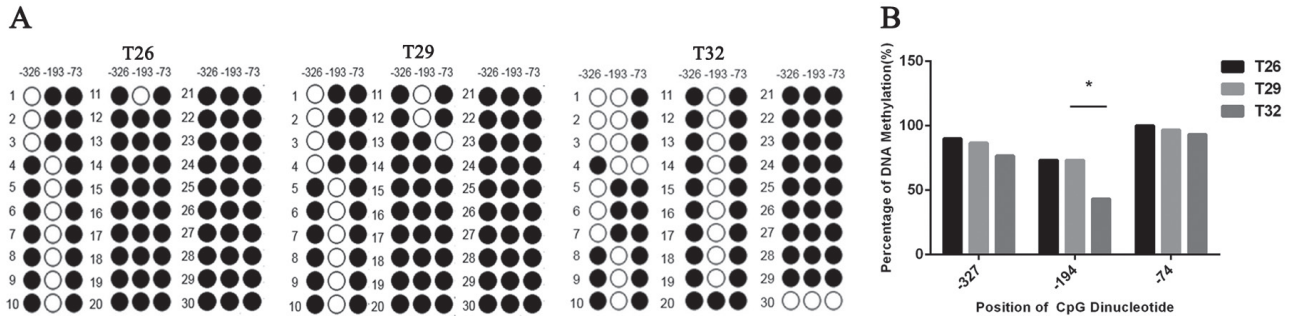

Figure 3 The effect of temperature on DNA methylation level of the *cyp19a1* promoter. (A) DNA methylation patterns of the *cyp19a1* promoter at different incubation temperatures. Each line represents one clone (30 clones/temperature) and the column numbers indicate CpG positions relative to the transcription starting site. Open and closed circles denote unmethylated and methylated CpG sites, respectively. (B) The percentage of DNA methylation at each CpG site at different incubation temperatures. Asterisk indicates a significant difference (P<0.05).

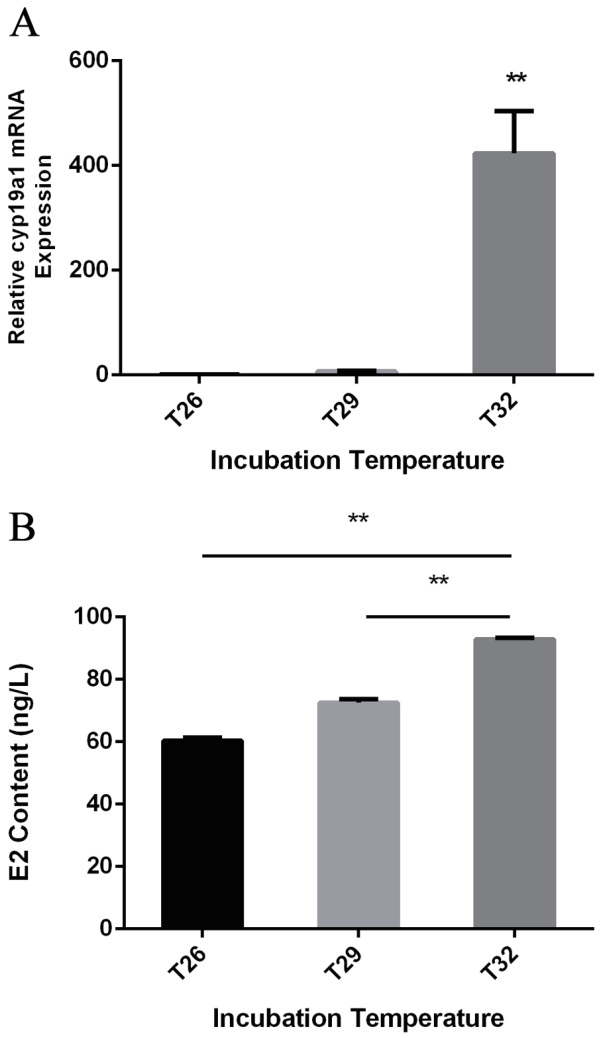

Figure 4 Relative expression of the *cyp19a1* gene and estrogen in the AKG complexes at different incubation temperatures. (A) Quantitative RT-PCR results for the *cyp19a1* gene at different temperatures. (B) Quantitative analysis of estrogen content in the AKG complexes using ELISA. Values are shown as mean ± standard error. Double asterisk indicates a highly significant difference (P<0.01).

in Reeves' turtle was identified, and bisulfite sequencing showed that a high incubation temperature led to de-methylation in the promoter region. There was also a significant correlation between de-methylation and the increased gene expression of *cyp19a1*.

The results are consistent with research on the European sea bass and the red-eared slider turtle (Navarro-Martín *et al.*, 2011), indicating that de-methylation at the promoter region of *cyp19a1* gene is responsible for a high expression of *cyp19a1* at female-biased temperatures. Consistent with the function of *cyp19a1*, the increased gene expression of *cyp19a1* correlates with an increased estrogen content. Sex steroid hormones have been proved to be the trigger for sex determination in turtles (Crews, 1996). Endogenous estrogen can block gonadal masculinization of slider turtle (*Trachemys scripta elegans*) eggs incubated at a male-producing temperature, providing evidence that estrogen is capable of overriding the effect of incubation temperature during the TSP (Crews *et al.*, 1991). In addition, administration of estrogen to the gonads of sea turtles incubated at male-producing temperatures resulted in up-regulation of *FoxL2* and aromatase and down-regulation of testicular factor Sox9, indicating that estrogen has a feedback effect on sex-related gene expression during gonad development (Díaz-Hernández *et al.*, 2015). Our results showed that AKG estrogen content depends on the incubation temperature of the eggs during the TSP, and correlates with the sex ratios in Reeves' turtle. Therefore, we hypothesized that variation in methylation levels of *cyp19a1* gene induced by temperature, leading to changes in *cyp19a1* gene expression levels and estrogen content, may be a vital regulation mechanism for TSD in Reeves' turtles.

In this study, we also predicted several transcription factor binding sites (TFBSs) related to sex determination

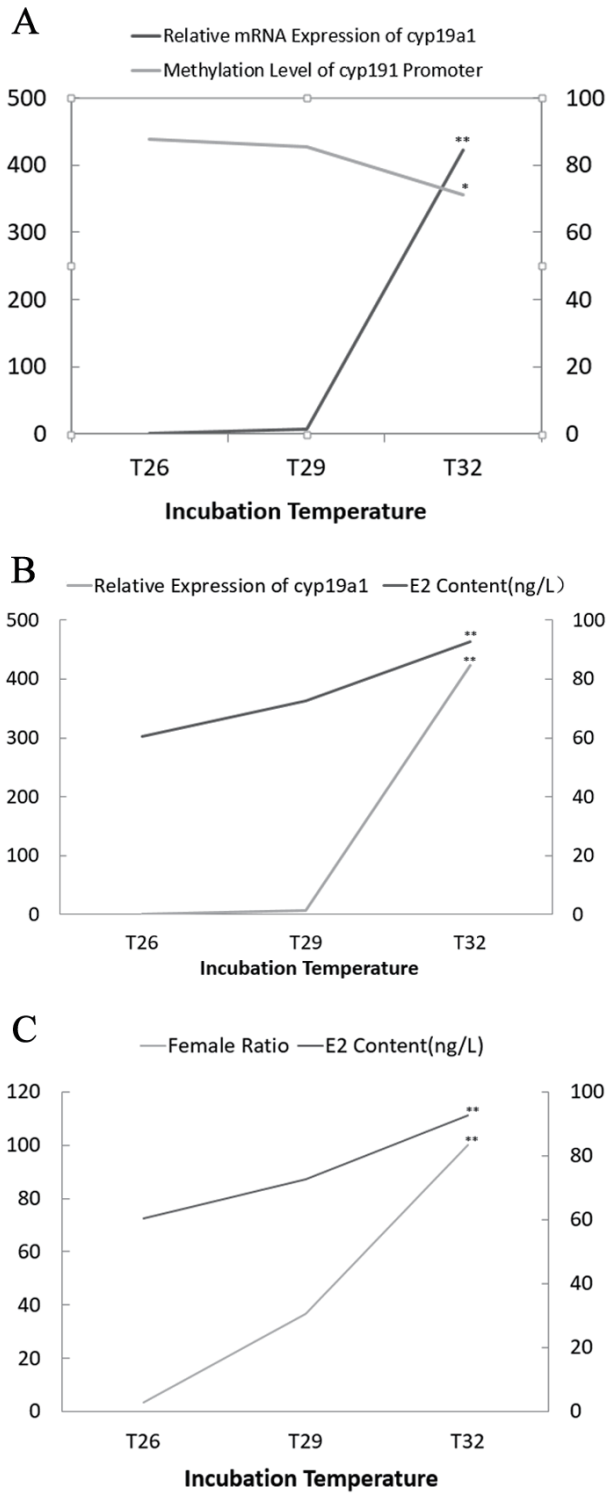

Figure 5 Correlation between the DNA methylation level of the *cyp19a1* promoter, expression of *cyp19a1*, estrogen content and sex ratio. (A) The correlation between the DNA methylation of the *cyp19a1* promoter and the expression of *cyp19a1*. (B) The correlation of *cyp19a1* relative expression and estrogen content under different temperature treatments. (C) The correlation of estrogen levels and female hatchling ratios under different temperature treatments. Double asterisks and single asterisk indicate significant differences at $P<0.01$ and $P<0.05$, respectively.

on the 5'-flanking region of the *cyp19a1* gene sequence. For example, in vitro studies show that either *SF-1* or *FoxL2* can enhance the expression of the *cyp19a1* gene, but simultaneous co-transfection of *SF-1* and *FoxL2* with the *cyp19a1* promoter constructs have an additive effect on gene expression (Nakamoto *et al.*, 2007; Wang *et al.*, 2007). The transcription factor Dmrt1 can directly repress *cyp19a1* gene transcription and estrogen production in the gonads of tilapia (Wang *et al.*, 2010). It is worth noting that the CpG site at –163 bp, near the FOX binding site, showed significantly lower methylation at high incubation temperatures, similar to the observations for red-eared slider turtles, suggesting that a low methylation level may allow *FoxL2* to bind to this site at high temperatures, contributing to transcriptional activation of *cyp19a1* (Navarro-Martín *et al.*, 2011). However, further research is needed to support this speculation.

Taken together, the data obtained from assays carried out on AKG tissues demonstrate that the de-methylation response of the *cyp19a1* gene to incubation temperature, especially at female-biased temperatures, could lead to the temperature-specific expression of aromatase and estrogen, which may further determine gonadal development in Reeves' turtles.

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